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Production and Characterization of Extracellular Phospholipase D from *Streptomyces* sp. YU100

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Abstract Using *Streptomyces* sp. YU100 isolated from Korean soil, the fermentative production of phospholipase D was attempted along with its purification and characterization studies. When different carbon and nitrogen sources were supplemented in the culture medium, glucose and yeast extract were found to be the best. By varying the concentration of nutrients and calcium carbonate, the optimal culture medium was determined as 2.0% glucose, 1.5% yeast extract, 0.5% tryptone, and 0.3% calcium carbonate. During cultivation, the strain secreted most of the phospholipase D in the early stage of growth within 24 h. The phospholipase D produced in the culture broth exhibited hydrolytic activity as well as transphosphatidylation activity on lecithin (phosphatidylcholine). In particular, the culture broth showed 8.7 units/ml of hydrolytic activity when cultivated at 28°C for 1.5 days. The phospholipase D was purified using 80% ammonium sulfate precipitation and DEAE-Sepharose CL-6B column chromatography, which produced a major band of 57 kDa on a 10% SDS-polyacrylamide gel with purity higher than 80%. The enzyme showed an optimal pH of 7 in hydrolytic reaction, and at pH 4 in a transphosphatidylation reaction. The enzyme activity increased until the reaction temperature was elevated to 60°C. The enzyme was relatively stable at high temperatures and neutral pH, but significantly unstable in the alkaline range. Among the detergents tested as emulsifiers of phospholipids, the highest enzyme activity was observed when 1.5% Triton X-100 was employed. However, no inhibitory effect by metal ions was detected. Under optimized reaction conditions, the purified enzyme not only completely decomposed PC to phosphatidic acid within 1 h, but also exhibited higher than 80% conversion rate of PC to PS by transphosphatidylation within 4 h.

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Phospholipase D is an industrially important enzyme for the production of certain functional phospholipids including phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol from lecithin (phosphatidylcholine; PC), a byproduct in soy-oil productions. These phospholipids are known to have some benefits for age-associated memory impairment [5] and mood enhancement from stress [19-20] due to structural and functional changes in the neuronal membranes in the lipid composition of the human brain [23]. In particular, significant improvements have been reported in the brain functions of elderly patients with Alzheimer's disease [1], Parkinson's disease or dementia [6], epilepsy [16], and geriatric depression [3, 18].

The enzyme phospholipase D has two catalytic properties: one is the hydrolysis of lecithin to phosphatidic acid and choline, while the other is the transphosphatidylation of lecithin to functional phospholipids by the interconversion of the polar headgroups. In spite of its wide distribution in animals, plants, and microorganisms [15], microbial phospholipase D, especially from actinomycetes, has been extensively investigated due to its high transphosphatidylation activity compared to hydrolytic activity [25, 28].

Until now, several *Streptomyces* species have been investigated for their industrial application in the production of functional phospholipids, including *S. hachijoensis* [22], *S. lydicus* [26], *S. antibioticus* [24], *S. chromofuscus* [12], and *Streptoverticillium cinnamoneum* [21]. Recently, a new strain, *Streptomyces* sp. YU100 was isolated from Korean soil by the Institute of Biotechnology, Yeungnam University [14]. Accordingly, the current study investigated the fermentative profile of phospholipase D from this strain

along with its reaction characteristics in order to study a possibility of its industrial application.

MATERIALS AND METHODS

Reagents

The standard phospholipids including L-α-phosphatidycholine (PC; Type XVI-E, 99% pure, from fresh egg yolk) and L-α-phosphatidyl-L-serine (PS; 98% pure, from soybeans), as well as the choline oxidase and peroxidase used in the enzymatic analysis were purchased from Sigma Chemical Co., MO, U.S.A. All other reagents used in this work were chemical pure grades.

Fermentative Production of Phospholipase D by *Streptomyces* sp. Y100

The *Streptomyces* sp. Y100 was maintained on Bennett's agar medium (1.0% glucose, 0.1% yeast extract, 0.2% peptone, 0.1% beef extract, pH 7.0). The fermentative production of phospholipase D was carried out on a typical production medium consisting of 1.5% yeast extract, 0.5% peptone, 2.0% glucose, and 0.3% calcium carbonate (pH 7.2), and cultivated at 28°C for 3 days with shaking at 200 rpm. In order to determine the optimal medium composition, various carbon and nitrogen sources in different concentrations were supplied in the production medium.

Determination of Enzyme Activity

The hydrolytic activity of phospholipase D was determined by measuring the amount of choline released from PC according to previous reports [11, 14, 26]. The typical reaction mixture consisted of 0.1 ml of PC emulsion (10 ml of distilled water was added to 500 mg of PC dissolved in 1 ml of diethyl ether and then sonicated on an ice bath), 0.1 ml of 0.1 M sodium citrate-phosphate buffer (pH 7.0), 0.05 ml of 0.1 M calcium chloride, 0.15 ml of 5.0% Triton X-100, and 0.1 ml of the enzyme, and the mixture was then incubated at 37°C for 10 min. One unit of enzyme was defined as the amount required to release 1 μmol of choline from PC at 37°C for 1 min.

The transphosphatidylation reaction was carried out at 25°C in 2.0 ml of a reaction solution containing 1 μmol of PC in diethyl ether, 2 mmol L-serine in 0.2 ml of 0.2 M sodium citrate buffer (pH 4.0), and 0.2 ml of the enzyme solution. The reaction mixture was then analyzed on a high performance thin layer chromatography (HPTLC) plate (Silicagel 60 F_{254}, Merck KGaA, Germany), after being extracted with diethyl ether : ethanol (4 : 1, v/v) [10, 14, 26]. In this reaction, the average molecular weight of PC from egg yolk was estimated as 767, based on the fatty acid composition of egg yolk reported by Gurr and Harwood [8].

Purification of Phospholipase D

The culture broth of *Streptomyces* sp. YU100 was first treated with 80% ammonium sulfate, and the precipitate was then recovered by centrifugation at 12,000 rpm for 15 min. After being dissolved in 25 mM Tris buffer (pH 8.0), the solution was dialyzed against the same buffer. Next, the enzyme solution was loaded on a DEAE-Sepharose CL-6B ion exchange column (Amersham Pharmacia Biotech AB, Sweden), which was preequilibrated with 25 mM Tris buffer (pH 8.0). The column was eluted with a gradient of 1 M sodium chloride in the same buffer. One ml fractions were collected at a flow rate of 2 ml/min, while simultaneously recording absorbance at 280 nm.

Characterization of Phospholipase D

To examine the effects of pH on the enzyme activity and stability, 0.1 M sodium citrate-phosphate buffer within a range of pH 3 to 7 and 0.1 M Tris-HCl buffer within a range of pH 7 to 10 were employed in the reaction mixture. To study the enzyme stability at different pHs, the enzyme solution was kept in different buffers at 4°C for 24 h, dialyzed again with 0.1 M sodium citrate-phosphate buffer (pH 7.0), and then subjected to the reaction. At various temperatures, both hydrolysis and transphosphatidylation reactions were carried out for 10 min in order to find the optimal temperature. The thermal stability of the enzyme was also studied by measuring the enzyme activity at different temperatures. In order to test the effects of detergents and metal ions on the enzyme activity, detergents such as Triton X-100 and metal ions including Mn^{2+}, Zn^{2+}, or Co^{2+} were also added to the reaction mixture.

Analytical Procedures

The dry cell weight of *Streptomyces* sp. YU100 was weighed after being treated with 0.1 M hydrogen chloride and then dried at 80°C overnight on an aluminum plate. The protein content was measured by the Lowry method [17] using bovine serum albumin as the standard. A 10% SDS-polyacrylamide gel was also run to identify the protein band, stained with 0.1% Coomassie brilliant blue R-250, and destained with 10% methanol : 10% acetic acid (1 : 1, v/v) [2].

RESULTS AND DISCUSSION

Effect of Nutrients on Production of Phospholipase D by *Streptomyces* sp. YU100

As for the cultural characteristics of *Streptomyces* sp. YU100, it was found that the strain grew very well on ISP 2 and Bennett's agar plates, which contained glucose as the carbon source [14]. Thus, 2.0% of several different types of carbon sources were supplied, and their effects on the cell growth and production of phospholipase D were examined (Table 1). The strain grew on the carbon sources

Table 1. Effect of various carbon sources on production of phospholipase D from *Streptomyces* sp. YU100[1].

Carbon source	Final pH	Dry cell weight (g/l)	Produced phospholipase D (unit/ml)
Soluble starch	9.07	3.32	2.11
Maltose	8.99	3.58	4.52
Sucrose	9.11	3.20	2.92
Lactose	9.06	3.76	4.56
Glucose	7.39	5.82	6.61
Fructose	8.10	5.16	5.36
Glycerol	8.01	3.60	4.10

[1]*Streptomyces* sp. YU100 was inoculated into a medium containing a 2.0% carbon source, 2.0% yeast extract, and 0.3% calcium carbonate (pH 7.2) and cultivated at 28°C for 3 days with shaking at 200 rpm. The values are the mean of triple experiments.

tested, yet monosaccharides, including glucose and fructose, were better than glycerol, disaccharides, or polysaccharides for growth and enzyme production. Although higher enzyme production of some lipid metabolizing enzymes on glycerol medium than glucose medium has been previously reported [9, 28], glycerol did not increase the production of phospholipase D in the current strain.

In addition to yeast extract in the ISP 2 and Bennett's agar media, 2.0% several different nitrogen sources was tested for growth and enzyme production of the strain. As shown in Table 2, a higher biomass production was observed in complex nitrogen sources including yeast extract, tryptone, and peptone, whereas simple nitrogen sources including amino acid, nitrite, or nitrate resulted in very poor growth. This was also consistent with a previous report [14] that the current strain grows very poorly in glucose asparagine media and glucose nitrate media. Due to its effective growth in yeast extract and tryptone, the effect of their combination on the enzyme production was also investigated (Fig. 1). An increase in the enzyme production of as high as 25% was observed when the ratio of yeast extract/tryptone supplemented in the fermentation medium was 3 : 1.

Table 2. Effect of various nitrogen sources on production of phospholipase D from *Streptomyces* sp YU100[1].

Nitrogen source	Final pH	Dry cell weight (g/l)	Produced phospholipase D (unit/ml)
Yeast extract	7.40	5.86	6.69
Peptone	8.17	3.22	2.12
Tryptone	7.85	4.72	5.31
Urea	8.69	2.70	0.35
Glycine	8.22	1.88	0.48
$NaNO_2$	8.16	2.44	0.55
$NaNO_3$	7.98	1.88	0.07

[1]*Streptomyces* sp. YU100 was inoculated into a medium containing 2.0% glucose, 2.0% of a nitrogen source, and 0.3% calcium carbonate (pH 7.2) and cultivated at 28°C for 3 days with shaking at 200 rpm. The values are the mean of triple experiments.

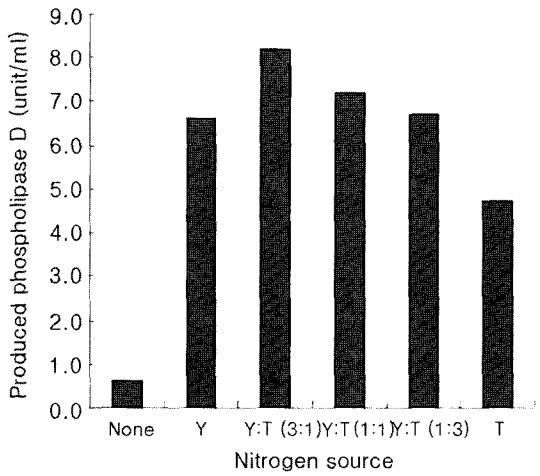

Fig. 1. Effect of combination of two nitrogen sources on production of phospholipase D in *Streptomyces* sp. YU100.
Streptomyces sp. YU100 was inoculated into a medium containing 2.0% glucose, 2.0% nitrogen source, and 0.3% calcium carbonate (pH 7.2), and cultivated at 28°C for 3 days with shaking at 200 rpm. As the nitrogen source, different combinations of yeast extract and tryptone were supplied in the medium for *Streptomyces* sp. YU100. Y, 2.0% yeast extract only; Y : T (3 : 1), 3 : 1 mixture of yeast extract and tryptone; Y : T (1 : 1), 1 : 1 mixture of yeast extract and tryptone; Y : T (1 : 3), 1 : 3 mixture of yeast extract and tryptone; T, 2.0% tryptone only.

Fermentative Production of Phospholipase D by *Streptomyces* sp. YU100

In optimized culture media (2.0% glucose, 1.5% yeast extract, 0.5% tryptone, 0.3% calcium carbonate, pH 7.2), the fermentative production of phospholipase D was investigated

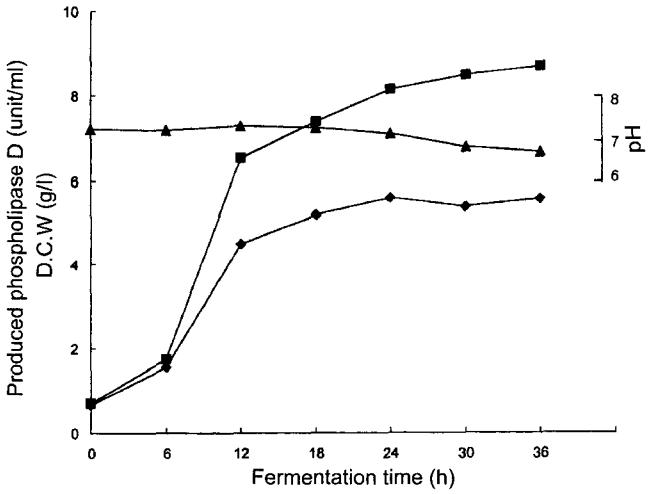

Fig. 2. Fermentation profiles for production of phospholipase D from *Streptomyces* sp. YU100.
Streptomyces sp. YU100 was inoculated into a medium containing 2.0% glucose, 1.5% yeast extract, 0.5% tryptone, and 0.3% calcium carbonate (pH 7.2), and cultivated at 28°C for 3 days with shaking at 200 rpm. The values are means of triple experiments. ◆, Dry cell weight (D.C.W.); ▲, pH of culture broth; ■, produced phospholipase D.

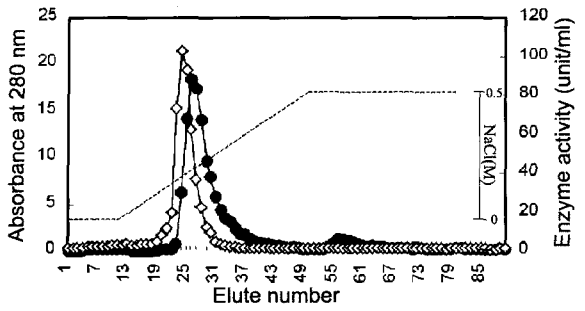

Fig. 3. Elution profile of phospholipase D on DEAE-Sepharose CL-6B ion exchange column chromatography.
The enzyme solution recovered from 80% ammonium sulfate precipitation was dissolved in 25 mM Tris buffer (pH 8.0), and then loaded onto a DEAE-Sepharose CL-6B ion exchange column (2.5×20 cm) equilibrated with the same buffer. Elution was carried out with a gradient of 1 M sodium chloride at a flow rate of 2 ml/min. ●, Absorbance at 280 nm; ◇, phospholipase D activity.

in a 300-ml batch culture system. The amount of extracellular phospholipase D produced (hydrolytic activity) was as high as 8.7 unit/ml of culture broth after 3 days of cultivation, as seen in Fig. 2. This value is much higher than the productivity of *Streptoverticillium cinnamoneum* (1.85 unit/ml) [7] and that of *S. antibioticus* or its recombinant *Escherichia coli* strain (ca. 5 unit/ml) [13], but similar to that of *Streptomyces* sp. of Shon *et al.* (8.3 unit/ml) [28] and somewhat lower than that of *S. lydicus* (11.5 unit/ml) [27].

In addition, it was observed that the production of phospholipase D was closely associated with the cell growth. This is a common feature in phospholipase D production in *Streptomyces* species, thereby strongly suggesting that most of the phospholipase D produced was secreted into the culture broth [27-28]. Even though there was no significant change in pH during the fermentation, it is not clear whether or not this could be attributed to the calcium carbonate supplemented in the culture broth [28].

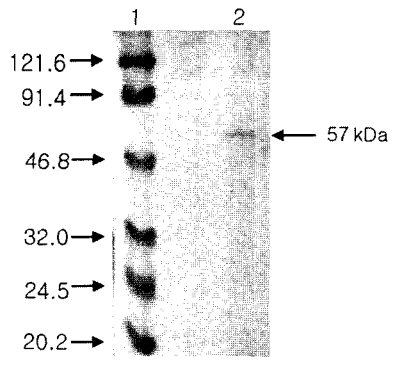

Fig. 4. Eletrophoretic profile of partially purified phospholipase D of *Streptomyces* sp. YU100.
The partially purified phospholipase D was subjected to 10% SDS-polyacrylamide gel electrophoresis. Lane 1, size marker; lane 2, purified phospholipase D.

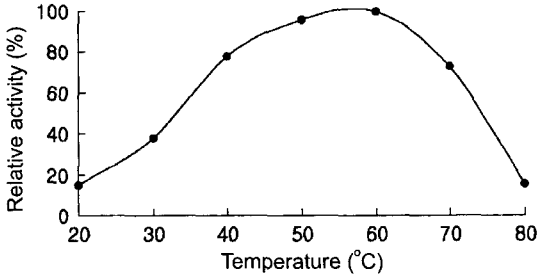

Fig. 5. Effect of temperature on hydrolytic activity of phospholipase D of *Streptomyces* sp. YU100.
The reaction mixure consisted of 0.1 ml of PC emulsion, 0.1 ml of 0.1 M sodium citrate-phosphate buffer (pH 7.0), 0.05 ml of 0.1 M calcium chloride, 0.15 ml of 5.0% Triton X-100, and 0.1 ml of the enzyme, and was incubated at different temperatures for 10 min. The values are means of triple experiments.

Partial Purification of Phospholipase D of *Streptomyces* sp. YU100

The phospholipase D of *Streptomyces* sp. YU100 was purified from the culture broth by 80% ammonium sulfate, followed by DEAE-Sepharose CL-6B ion exchange chromatography (Fig. 3). The specific activity of the purified enzyme was 33 unit/mg of protein, and its purification fold was 17.6. In 10% SDS-polyacrylamide gel electrophoresis, the molecular weight of the enzyme in the major band was estimated as 57 kDa (Fig. 4), yet the purified enzyme preparation showed only 80% purity in density. Considering its molecular weight, the phospholipase D from *Streptomyces* sp. YU100 can be included in the same category as other phospholipase D's purified from actinomycetes, as summarized in Table 4.

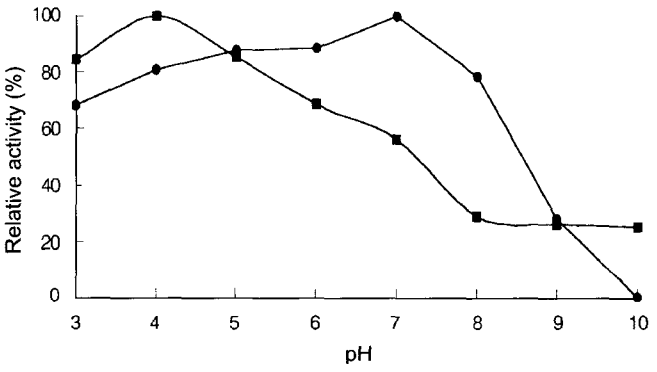

Fig. 6. Effect of pH on activity of phopholipase D of *Streptomyces* sp. YU100.
The hydrolysis reaction mixture consisted of 0.1 ml of PC emulsion, 0.1 ml of 0.1 M buffer, 0.05 ml of 0.1 M calcium chloride, 0.15 ml of 5.0% Triton X-100, and 0.1 ml of the enzyme, which was incubated at 37°C for 10 min. The transphosphatidylation reaction was carried out in 2.0 ml of a reaction solution containing 1 μmol of PC in diethyl ether, 2 mmol of L-serine in 0.2 ml of 0.2 M buffer, and 0.2 ml of the enzyme solution at 25°C for 10 min. A sodium citrate-phosphate buffer was used within the pH 3 to 7 range, and Tris-HCl buffer within of pH 7 to 10. The values are means of triple experiments. ●, Hydrolytic activity; ■, transphosphatidylation activity.

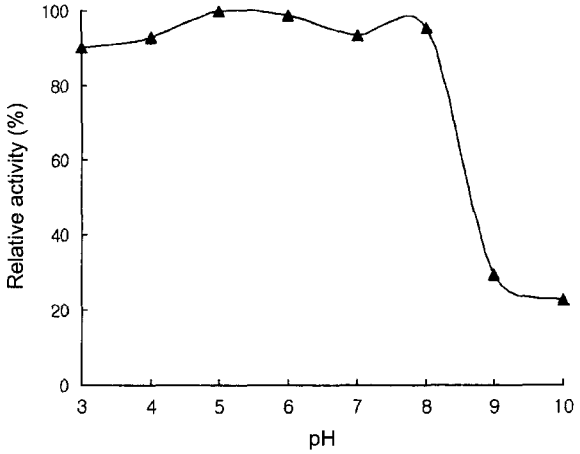

Fig. 7. pH stability of phospholipase D of *Streptomyces* sp. YU100.

The purified enzyme solution was stored in each buffer at 4°C for 24 h, dialyzed again with 0.1 M sodium citrate-phosphate buffer (pH 7.0), and then included in the reaction mixture. The enzyme activity was measured in a reaction mixture of 0.1 ml of PC emulsion, 0.1 ml of 0.1 M sodium citrate-phosphate buffer (pH 7.0), 0.05 ml of 0.1 M calcium chloride, 0.15 ml of 5.0% Triton X-100, and 0.1 ml of the enzyme at 37°C for 10 min. The values are means of triple experiments.

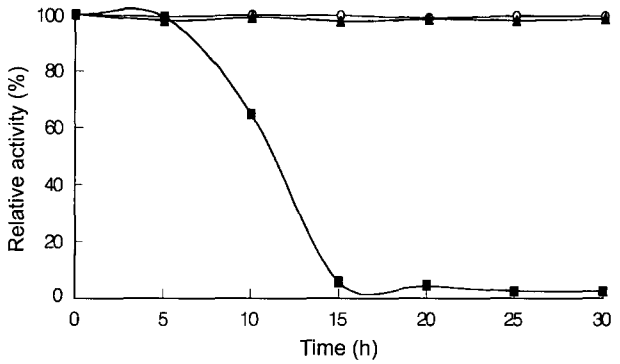

Fig. 8. Thermal stability of phospholipase D of *Streptomyces* sp. YU100.

The purified enzyme solution was immersed in a water bath at different temperatures, and a sample of the enzyme was taken at time intervals and included in the reaction mixture. The enzyme activity was measured in a reaction mixture of 0.1 ml of PC emulsion, 0.1 ml of 0.1 M sodium citrate-phosphate buffer (pH 7.0), 0.05 ml of 0.1 M calcium chloride, 0.15 ml of 5.0% Triton X-100, and 0.1 ml of the enzyme at 37°C for 10 min. The values are the mean of triple experiments. ○, 37°C; ▲, 60°C; ■, 80°C.

Effects of pH and Temperature on the Activity and Stability of Phospholipase D

Using the partially purified phospholipase D from *Streptomyces* sp. YU100, its reaction characteristics and enzyme stability were examined. The optimum reaction temperature was 60°C for the hydrolysis of PC (Fig. 5), and the optimum reaction pH was observed as 7 (Fig. 6). This is a pattern similar to other phospholipase D's from actinomycetes.

However, it was striking that the optimal pH of the enzyme for a transphosphatidylation reaction was 4 (Fig. 6), as distinct from the phospholipase D of *S. antibioticus* [24] or *S. cinnamoneum* [21], where the optimal pH for transphosphatidylation reaction is nearly the same as that for the hydrolytic reaction. As such, it would appear that a higher transphosphatidylation yield from PC to PS can be achieved by the current enzyme within the acidic pH range, which may provide some benefits in its industrial application.

When investigating the enzyme stability at various pHs, *Streptomyces* sp. YU100 phospholipase D exhibited high stability within a range from acidic to neutral pH (3–8), but lost its activity above pH 8 (Fig. 7). Similar results have been reported for the phospholipase D from *S. lydicus* [25] and *Streptomyces* sp. PMF and PM43 [4], yet these enzymes were inactivated at pH 3, implying that the enzyme from *Streptomyces* sp. YU100 is more stable within an acidic pH range.

As seen in Table 4, the phospholipase D from *Streptomyces* sp. YU100 also exhibited high thermal stability up to 60°C (Fig. 8), similar to other phospholipase D's from actinomycetes. In particular, the current enzyme kept its activity at 37°C without any loss for 24 h, and more than 90% of its activity remained at 60°C after 24 h.

Table 3. Effect of detergents on the hydrolytic activity of phospholipase D of *Streptomyces* sp. YU100[1].

Biological detergent	Ionic	Relative activity (%)
Deoxycholic acid	Anionic	17.6
N-Lauroyl-sarcosine	Anionic	8.1
Lauryl sulfate	Anionic	9.0
CHAPS	Zwitter-ionic	14.2
Nonidet P-40	Non-Ionic	20.0
Polyoxyethylene 4 laury ether (Brij 30)	Non-Ionic	28.7
Polyoxyethylene sorbitan monolaurate	Non-Ionic	14.9
Triton X-100	Non-Ionic	100.0
None		17.2

[1]The reaction mixture consisted of 0.1 ml of a PC emulsion, 0.1 ml of 0.1 M sodium citrate-phosphate buffer (pH 7.0), 0.05 ml of 0.1 M calcium chloride, 0.1 ml of the enzyme, and 1.5% detergent, and incubated at 37°C for 10 min. The values are the mean of triple experiments.

Table 4. Comparison of properties of phospholipase D isolated from actinomycetes.

Strain	Molecular mass (kDa)	Activity (unit/mg)	Optimum pH		Optimum temp. (°C) (hydrolysis)	Ref.
			Hydrolysis	Transphosphatidylation		
S. sp. YU100	57	33	7.0	4.0	60	This work
S. hachijoensis	16	631	7.5	-[1]	50	[22]
S. lydicus	56	2390	5.5	-[1]	60	[26]
S. antibioticus	64	1436	5.5	6.0	-[1]	[24]
S. chromofuscus	57	152	5.0	-[1]	50	[12]
S. sp. PMF	54	42	5.0	-[1]	60	[4]
S. sp. PM43	54	45	6.5	-[1]	60	[4]
S. cinnamoneum	54	468	5.0	5.0	50	[21]

[1]Columns denoted as '-' means no data available.

Effects of Detergents and Metal Ions on Activity of Phospholipase D

Because the substrate PC is insoluble in water, certain detergents have been employed in hydrolysis and transphosphatidylation reactions as emulsifiers [12]. As shown in Table 3, supplementation of 1.5% Triton X-100 in the reaction medium increased the hydrolytic activity of the enzyme by as much as 5.8 times, compared to the case without Triton X-100. No other detergents produced any significant increment in the enzyme activity, even though deoxycholic acid was previously reported to be a good emulsifier in the reaction of the phospholipase D from *S. chromofuscus* [12]. It was also interesting to find that all the ionic detergents, except for deoxycholic acid, inhibited the hydrolytic activity of the enzyme.

Metal ions such as Ca^{2+}, Mg^{2+}, and Al^{3+} have been previously reported to increase the activity of some phospholipase D's from actinomycetes, whereas Mn^{2+}, Zn^{2+}, and Co^{2+} slightly inhibit the activity [12, 21-22]. Yet, when metal ions such as Mn^{2+}, Zn^{2+}, or Co^{2+}, as well as EDTA or EGTA as chelating agents, were supplemented in the hydrolytic reaction mixture, no significant effects were observed in the phospholipase D from *Streptomyces* sp. YU100 (data not shown).

Reaction Kinetics of Phospholipase D from *Streptomyces* sp. YU100

Under optimized reaction conditions, the conversion profiles for hydrolysis and transphosphatidylation by phospholipase D from *Streptomyces* sp. YU100 were analyzed. In the hydrolysis reaction, PC was completely decomposed to phosphatidic acid by the enzyme within 1 h. In contrast, the transphosphatidylation reaction of PC to PS proceeded for 4 h with a higher than 80% conversion rate (Fig. 9).

In conclusion, the phospholipase D from *Streptomyces* sp. YU100 exhibited reaction characteristics somewhat different from other actinomycetes phospholipase D's (Table 4). In particular, the difference in the optimal pH for

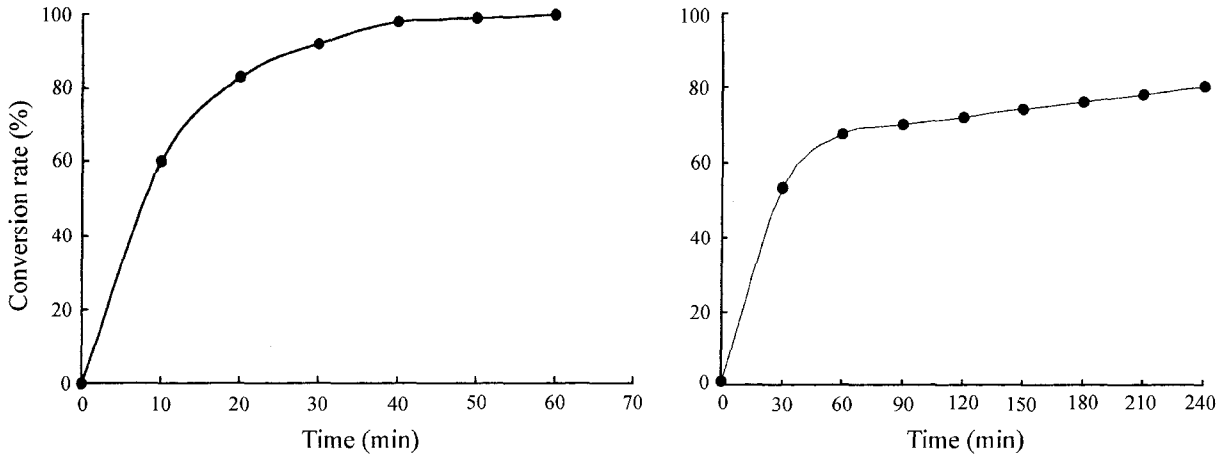

Fig. 9. Conversion profile of phosphatidylcholine by phospholipase D of *Streptomyces* sp. YU100.
(A) The hydrolysis reaction was carried out at 37°C in a mixture of 0.1 ml of PC emulsion, 0.1 ml of 0.1 M sodium citrate-phosphate buffer (pH 7.0), 0.05 ml of 0.1 M calcium chloride, 0.15 ml of 5.0% Triton X-100, and 0.1 ml of the enzyme, and the conversion rate was calculated as [choline produced]/[initial phosphatidylcholine]. (B) The transphosphatidylation reaction was carried out at 25°C in 2.0 ml of a reaction solution containing 1 μmol of PC in diethyl ether, 2 mmol of L-serine in 0.2 ml of 0.2 M sodium citrate buffer (pH 4.0), and 0.2 ml of the enzyme solution, and the conversion rate was calculated by measuring the density of the phosphatidylcholine and phosphatidylserine spots on a HPTLC plate.

hydrolysis and transphosphatidylation reaction will make the current enzyme more promising for industrial application in the production of functional phospholipids.

Acknowledgments

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